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Advances in Applied Science Research, 2013, 4(3):39-45

Advances in Applied Science Research
Pelagia Research Library

ISSN: 0976-8610
CODEN (USA): AASRFC

A protocol for rapid propagation of genetically *true to type* Indian turmeric (*Curcuma longa* L.) through *in vitro* culture technique

Arghya Ghosh[1,2], Padma Chatterjee[1] and Parthadeb Ghosh[2]*

[1]*Plant Biochemistry, Molecular Biology & Advance Plant Physiology Research Laboratory , Department of Botany, University of Kalyani, Kalyani, Nadia, West Bengal*
[2]*Cytogenetics & Plant Breeding Section, Department of Botany, University of Kalyani, Kalyani, Nadia, West Bengal*

ABSTRACT

As the plant Curcuma longa L. is an ethnomedicinally important one and almost all the parts of the plant are reported to contain curcumin and its structural analogues. Rhizomatous explants from two months old buds were cultured on Murashige and Skoog's (MS) medium supplemented with different concentrations of cytokinins and auxins. During the first culture on 2.5 mg/l of 6-benzylamino purine (BAP) and 1.5 mg/l of α Napthalenic acetic acid (NAA) yeilds 9.00±0.57[a] number of shoots with an average shoot length of 7.20±1.01[a] cm. The elongated shoots produced 9.66±1.20[a] roots on half strength MS liquid medium supplemented with 2 mg/l of Indole 3 butyric acid (IBA) and showed 86 % survivability after hardening. Genetic fidelity of the micropropagated plantlets was confirmed using RAPD analysis employing 12 primers. This system provides high fidelity micropropagation system for efficient and rapid micropropagation of this important medicinal plant.

Key words: *Curcuma longa* L., micropropagation, genetic fidelity, RAPD

INTRODUCTION

The genus *Curcuma* L. of the family Zingiberaceae is well known as the turmeric genus, because of *Curcuma longa* L. *C. longa* is the most investigated species of this genus, although there are over 100 others in this genus [1]. Turmeric is conventionally propagated vegetatively through rhizome bits carrying one or two buds. Rich morphological and genetic diversity is observed among the cultivated types of turmeric, probably due to vegetative mutations accumulated over a period of time. The rarity of seed set hampers recombination breeding. In such circumstances, biotechnological tools gain relevance in solving many crop specific problems and for crop improvement. However, these efforts have been seriously constrained due to absence of well characterized germplasm for augmenting the need of gene pool in the genetic improvement programs. *In vitro* regeneration or micropropagation is the best alternative to overcome these hurdles and it holds tremendous potential for rapid multiplication and production of high quality medicines from them [2, 3, 4, 5, 6]. Hence, there is a need to develop *in vitro* germplasm of the wild and cultivated species of *Curcuma* in India. There is also a need to standardize high fidelity, rapid and reliable protocol for micropropagation of *Curcuma longa.* Many workers reported successful micropropagation of turmeric [7] among them Nadgauda was the first to report micropropagation of turmeric. In this paper we have also design an alternative protocol for clonal propagation of *C. longa* from rhizomal explant and assessment of its genetic fidelity through RAPD technique.

MATERIALS AND METHODS

Collection of explant

Rhizomes of *C. longa* was collected in the month of July 2010 from experimental garden of Department of Botany, University of Kalyani, which located at $22^0$57′ 92 N latitude, $88^0$22′ E longitude with an average altitude of 9.75 m above mean sea level. The plant was identified in the Taxonomy and Plant systematic Unit, Department of Botany, University of Kalyani, Nadia.

Explant preparation

Young disease free rhizomal explants (rhizomal buds of 2.5 - 3 cm) were collected from 2 months old plant. Explants were washed thoroughly under running tap water and then treated with 5% (m/v) Teepol (Qualigen, Mumbai, India) for 15 min, followed by rinsing three to five times in sterile double distilled water. Another round of disinfestation was done with 70% alcohol for 5 min followed by immersion in 0.1% (m/v) aqueous mercuric chloride ($HgCl_2$) solution for 5 - 6 min and finally rinsed with sterile double distilled water (five to six times) in a flow chamber. The surface sterilized explants were trimmed at cut ends and about 1.2-1.5 cm prior to inoculation on culture media.

Media and culture conditions

Surface sterilized rhizomal segments (1.2 - 1.5 cm) were cultured on MS [8] basal medium containing 3% (w/v) sucrose (Himedia, Mumbai, India) for culture initiation and served as explant sources for subsequent experiments. The pH of the medium was adjusted to 5.8 before gelling with 0.8% (w/v) agar (Himedia, Mumbai, India). The explants initially were implanted vertically on the culture medium in test tube (150 × 25 nm) and plugged tightly with non absorbent cotton. All the cultures were kept under cool fluorescent light (16 h photo period 40 µmol·m–2 s–1, Philips, India at 25°C ± 2°C) and 60% - 70 % relative humidity (RH).

Multiple shoot induction and elongation

For initial multiple shoot induction, the explants were cultured on MS medium [8] supplemented with various concentrations of BA (0.5 - 5.0 mg/l) in combination with NAA (0.5 - 5.0 mg/l). The induced shoots were allowed to grow for 22 days.

Rooting

Small micro shoots grown on subculture medium were transferred to half and full strength MS [8] media separately, supplemented with various concentrations of IBA (0.5 – 5 mg/l) for root developement. IBA was filter sterilized and added to the medium after autoclaving under the sterilized environment of laminar air flow cabinet. Data were recorded on the percentage of rooting, the mean number of roots per shoot and the root length after four weeks of transfer onto the rooting medium.

In vitro and *ex vitro* hardening of plantlets

The complete rooted plantlets with 7 - 9 fully expanded leaves were removed from the culture medium and the roots were washed gently under running tap water to remove agar. The plantlets were transferred to plastic pots (5 cm diameter) containing a mixture of sterilized garden soil and vermiculite in the ratio 2:1 and covered with transparent plastic bags to ensure high humidity. Each was irrigated with 1/6 MS basal salt solution devoid of sucrose and inositol every 4 days for 2 weeks. The growth chamber was maintained at 26°C ± 1°C, 80% - 85% relative humidity with light intensity of 50 µmol·m–2·s–1 on a 16 h photoperiod inside the culture room conditions. The relative humidity was reduced gradually and after 30 days the plantlets were transferred to pots (25 cm diameter) containing garden soil and kept under green house for another 2 weeks. There are no changes in respect to morphology, growth characteristics and floral features etc in between tissue culture regenarate plants and naturally grown field plants.

Statistical analysis

Experiments were set up in completely randomized block design. Each experiment was repeated three times with 10 - 12 replicates. Data were analyzed by one way analysis of variance (ANOVA) and the difference between means were scored using Duncan's Multiple Range Test $P \leq 0.05$ [9] on the statistical package of SPSS (Version 10).

DNA isolation and RAPD analysis

Genomic DNA was extracted from young leaves of *in vitro* raised and field grown plants of *Curcuma caesia* Roxb. and mother plant by Cytl trimethyl ammonium bromide (CTAB) procedure [10] with minor modifications. Quality

and quantity of DNA was checked on 0.8% agarose gel and also from values obtained by 260/280 nm UV absorbance ratio [11]. Twelve arbitrary decamer RAPD primers (Bengalore Genni Pvt. Ltd., India) were used for polymerase chain reaction (PCR) for DNA amplification. DNA finger printing profiles were compared to evaluate clonal fidelity and genetic stability. Amplification was performed in 25 µL using PCR mixture of consisting of 2.5 µL Taq buffer, 1 µL dNTPs, 0.5 µL Taq polymerase, 2 µL DNA (approximate 50 ng/µL), 1.0 µL primer (10 pmol), 2.5 µL $MgCl_2$, 1 µL oil and 14.5 µL MiliQ water. The PCR reaction conditions were: preheating for 5 min at 94°C; 40 cycles of 25 sec at 94°C, 20 sec at 40°C and 1.25 min at 72°C and elongation was completed by a final extension of 6 min at 72°C. After amplification, the PCR product was resolved by electrophoresis in 1.4% agarose gel (Himedia, Mumbai, India) and stained with ethidium bromide (0.5µg/ml). 2.0 - 23.1 kb λ DNA di-gested Hind III was used as the DNA marker, and bands were visualized under UV light and photographed using the Gel Doc equipment (Bio Rad). All the PCR reaction was repeated for thrice.

RESULTS AND DISCUSSION

In vitro establishment of explant

To overcome contamination problem, surface sterilization of explants was done with 0.1% aquous solution of Mercuric chloride ($HgCl_2$) for 2, 4, 6, 8 and 10 minutes. Mercuric chloride ($HgCl_2$) is a very strong sterilant [12]. When the explants sterilization was done with 0.1% aquous solution of $HgCl_2$ for 5 min, 60 % of the explants get survived. Whereas, exposure of 0.1% aquous solution of $HgCl_2$ above and below 5 minute prove to allow death or contamination of explant respectively.

A= Plants in the experimental garden, B= Germination from bud of rhizome, C= Shoot induction, D= Shoot elongation, E= Shoot multiplication, F= Clonally propagated shoot established in rooting medium, G= Induction of root with multiple shoot

Table 1: Standardization of 0.1% $HgCl_2$ for explant sterilization

Serial No.	Treatment duration (min) with 0.1% $HgCl_2$	Number of explants inoculated	Rate of contamination (after day of treatment)						Percentage of contamination free explants after 10 days
1	2	10	2	3	4	5	7	10	0
2	4	10	2	3	4	5	7	10	0
3	5	10	2	3	3	5	7	10	10'
4	6	10	2	3	3	6	6	9	60*
5	8	10	1	1	2	3	4	4	80**
6	10	10	0	0	1	1	1	2	90**

' = Death of inoculated explant due to contamination
** = Yeild of contamination free explants*
***= Brownish and death of inoculated explant due to long time exposure of 0.1% $HgCl_2$*

Induction and elongation of multiple shoots

When Explants were cultured on basal MS medium or, MS medium [8] contains solely cytokinin (BA), or auxin (NAA) failed to produce shoots even after 4 weeks of inoculation. MS medium supplemented with different concentrations and combinations of cytokinins and auxins showed variation in the regeneration percentage and number of shoots formed. Among the different combinations of cytokinin and auxin tested, the best response (81.66 ± 4.84^{a} %) was obtained in the presence of 2.5 mg/l BA and 1.5 mg/l NAA (Figure E, F, G) after 22 days of incubation. The average length of shoot in this medium was 7.20 ± 1.01^{a}. The BA and NAA concentrations higher than above concentrations of BA and NAA, the number of shoots as well as percent response was reduced (Table 2). This is probably due to higher concentration of nitrogen and potassium [13, 14, 15]. The stimulating effectivity of BA and NAA on multiple shoot formation has been reported earlier for several medicinal plant [16, 17, 18, 19, 20].

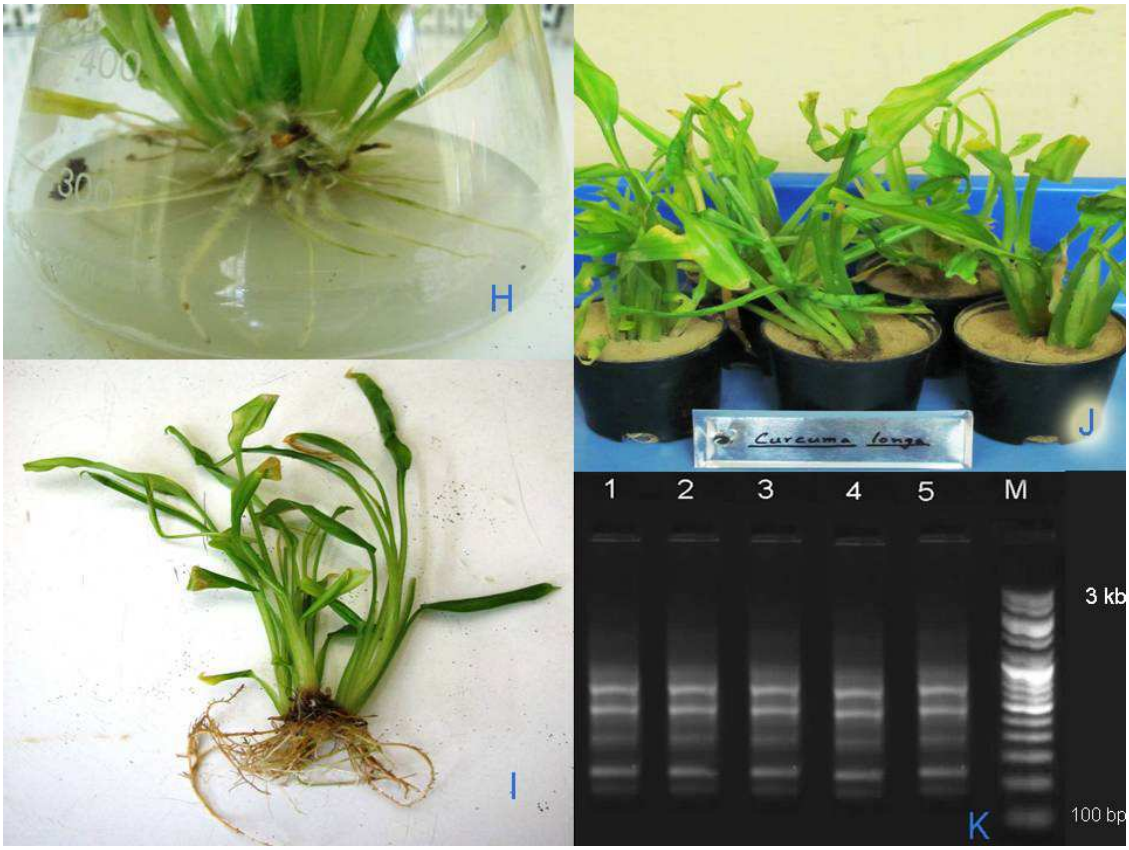
Table 2: Standardization of concentrations and combinations of BA and NAA for shooting in full strength MS media

Conc. of BA and NAA	% of response	No. of shoots/explant	Average shoot length
Basal MS (Control)	0.00 ± 0.00^{f}	0.00 ± 0.00^{g}	0.00 ± 0.00^{f}
MS+BA+NAA (mg/l)			
0.5+0.5	69.66 ± 3.71^{b}	7.33 ± 0.33^{abc}	5.33 ± 0.33^{bc}
0.5+1	54.00 ± 7.00^{cd}	7.66 ± 0.88^{abc}	5.16 ± 0.12^{bcd}
1+1	51.00 ± 3.05^{cd}	8.66 ± 1.45^{ab}	5.46 ± 0.24^{bc}
1.5+1	68.00 ± 2.08^{b}	7.33 ± 0.88^{abc}	5.76 ± 0.93^{b}
2+1	68.33 ± 2.33^{b}	6.33 ± 0.66^{cde}	5.80 ± 0.11^{b}
2.5+1.5	81.66 ± 4.84^{a}	9.00 ± 0.57^{a}	7.20 ± 1.01^{a}
3+2	61.33 ± 4.17^{bc}	6.66 ± 0.66^{bcd}	4.23 ± 0.39^{cd}
3.5+2	59.00 ± 3.05^{bc}	6.00 ± 0.57^{cde}	4.76 ± 0.39^{bcd}
4+2.5	61.33 ± 3.48^{bc}	4.33 ± 0.33^{ef}	3.83 ± 0.44^{d}
4.5+3	47.66 ± 2.72^{d}	5.00 ± 0.57^{def}	1.66 ± 0.33^{e}
5+4	34.00 ± 5.56^{e}	3.66 ± 0.66^{f}	2.26 ± 0.37^{e}

***Values are means ± SE. n = 10 - 12 (in triplicate); Means followed by same does not differ significantly according to Duncan's Multiple Range Test ($p \leq 0.05$).*

Induction of rooting

Healthy elongated shoots (4 - 9 cm in length) were excised and placed on both, full and half strength MS basal medium [8] supplemented with different concentrations of auxin (IBA) at the range of 0.5 - 5.0 mg /l for induction of roots (Table 3). In the preliminary experiments conducted, no rooting was observed when the shoots were culture on basal (Control) MS medium. Full strength MS medium containing auxins (IBA) showed very poor response in rooting even after 25 days, but well developed roots were achieved on half strength MS medium supplemented with IBA (2 mg/l) with increase sucrose concentration (4%) gave us well developed roots within 15 - 20 days [21, 12, 23]. In this medium shoot formed roots at a high frequency of 86.33 ± 6.11^{a} % and attaining an average length of 8.46 ± 0.37^{a} cm were obtained. Further increase in the IBA concentration leads to reduces root initiation [24].



H= Elongation of root, I= Mature clonally propagated plant with multiple shoot and root, J= Hardening of clonally propagated plants, K= RAPD analysis to detect genetic fidelity (lane 1, 2 contains genomic DNA from field plant & lane 3, 4, 5 contains genomic DNA from *in vitro* grown plant

Hardening

The well developed rooted plantlets were taken out gently from the test tubes and thoroughly washed with sterile water to remove adhered agar and traces of the medium to avoid contamination. The micropropagted plantlets were transferred to plastic pots containing potting a mixture of (2:1) soil and vermiculite (Figure J) in green house. Finally the acclimated plants were then shifted to the field conditions showing 86 % of survivility. The growth characteristics of *in vitro* raised plants were indentical in morphological with naturally occurring field plants.

RAPD analysis

There are many reports on molecular characterization of micropropagated plants by the RAPD technique especially to confirm the clonal fidelity and genetic stability among tissue culture grown plants and donor [25, 26, 27]. In this paper we have performed the genetic integrity of *in vitro* regenerated plants from rhizomal explants and respective naturally occurring field grown donor plant of *Curcuma longa* L.

Total 12 primers were initially screened and finally 6 primers produce clear and scorable amplified bands ranging from 3 - 5 bands per primer (Table 4). Each primer produced a unique set of amplification products ranging in size from 100 bp - 3 kb (Figure K with primer 5' CGGGATCCGC 3'). All 6 primers produced a total of 23 bands with an average of 3.84 fragments. All the scorable bands were monomorphic in nature, indicating homogeneity among the culture regenerates and genetic uniformity with that of the donor plants. The possible reason may be multiple shoot bud differentiation without intervening callus phase is least vulnerable to genetic changes. However, no differences were observed between mother plant and plantlets regenerated from rhizomal segments by any five primers tested in present RAPD study.

Table 3: Standardization of concentrations of IBA for rooting in half and full strength MS media

Rooting in MS half strength			
Basal MS ½ (Control)	% of response	No. of roots/explant	Average root length
MS½ +IBA (mg/l)	0.00±0.00^{k}	0.00±0.00^{i}	0.00±0.00^{j}
0.5	15.00±0.57^{j}	2.00±0.57^{ghi}	3.66±0.14^{efgh}
1	23.33±0.88^{ij}	5.66±0.33^{bcd}	5.56±0.21^{c}
1.5	34.66±0.88^{fgh}	4.66±0.33^{bcde}	7.00±1.00^{b}
2	86.33±6.11^{a}	9.66±1.20^{a}	8.46±0.37^{a}
2.5	49.00±3.78^{cd}	5.66±0.33^{bcd}	5.40±0.15^{cd}
3	48.33±6.00^{cd}	5.66±0.66^{bcd}	4.93±0.14^{cde}
3.5	45.66±2.90^{cde}	4.33±0.33^{cdef}	4.26±0.17^{cdef}
4	39.33±2.60^{efg}	6.33±0.33^{bc}	3.96±0.52^{ef}
4.5	32.00±2.64^{gh}	5.00±0.57^{bcde}	2.60±0.26^{ghi}
5	16.33±2.40^{j}	3.33±0.33^{defg}	2.40±0.66^{hi}
Rooting in MS full strength			
Basal MS (Control)	0.00±0.00^{k}	0.00±0.00^{i}	0.00±0.00^{j}
MS+IBA (mg/l)			
0.5	3.33±0.66^{k}	1.33±0.33^{hi}	4.16±0.26de^{f}
1	17.33±1.85^{j}	2.66±0.66^{efg}	4.60±0.23^{cdef}
1.5	51.66±2.84^{c}	5.33±0.88^{bcd}	4.40±0.20^{cdef}
2	51.00±1.15^{cd}	4.66±0.88^{bcde}	4.03±0.31^{def}
2.5	76.66±2.90^{b}	7.00±1.15^{b}	4.90±0.49^{cde}
3	80.33±3.52^{ab}	6.33±0.88^{bc}	3.33±0.33^{fghi}
3.5	51.66±0.88^{c}	4.33±0.88^{cdef}	3.83±0.68^{efg}
4	42.00±4.58^{def}	4.33±0.66^{cdef}	2.33±0.33^{hi}
4.5	35.00±2.30^{fgh}	3.66±1.20^{def}	3.80±0.72^{efg}
5	28.33±2.02^{hi}	4.00±1.00^{cdef}	2.00±0.40^{i}

***Values are means ±SE. n = 10 - 12 (in triplicate); Means followed by same does not differ significantly according to Duncan's Multiple Range Test ($p \leq 0.05$).*

Table 4: Number of amplification products generated with the use of RAPD primers to assess genetic fidelity of micropropagated and field grown plants

Serial No.	Primer Code	Nucleotide sequence (5'-3')	Number of generated bands
1	RAPD1	GTCCTACTCG	-
2	RAPD2	GTCCTTAGCG	3
3	**RAPD3**	**CGGGATCCGC**	**5**
4	RAPD4	CTTCCGGCAG	4
5	RAPD5	GGTATTACTT	4
6	RAPD6	TGGCTCGGTA	-
7	RAPD7	CTTCGGCAGA	-
8	RAPD8	GGTATTACTT	-
9	RAPD9	GACAATGGTA	-
10	RAPD10	TTAGCTTAGG	-
11	RAPD11	CTCTCCGCCA	3
12	RAPD12	GCACGCCGGA	4

CONCLUSION

In this present study, we have established an efficient and reliable micropropagation protocol for *in vitro* regeneration of *Curcuma longa* L. from rhizomal explant, which can ensure large scale propagation, as well as protocol can also be used for raising genetically uniform plants, which is important for the sustainable supply of plant materials to the pharmaceutical industries and for conservation of elite germplasm.

Acknowledgements
This work was supported by Grants in Aid from the University of Kalyani, Nadia, West Bengal.

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